



## Protection against heterologous *Streptococcus iniae* isolates using a modified bacterin vaccine in Nile tilapia, *Oreochromis niloticus* (L.)

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### Abstract

*Streptococcus iniae* is a significant pathogen impacting aquaculture production worldwide. The objectives of this study were to determine whether a developed modified *S. iniae* (ARS-98-60) bacterin vaccine is efficacious in Nile tilapia, *Oreochromis niloticus* (L.), against challenge with heterologous isolates from diverse geographical locations and to evaluate protein and antigenic variability among the isolates tested. Two groups of tilapia (approximately 5 g) were intraperitoneally (IP) vaccinated with 100 µL of the vaccine or sham vaccinated with 100 µL of sterile tryptic soy broth and held for 28 days. Fish were challenged with each isolate by IP injection of  $2-3 \times 10^7$  CFU per fish using calcein to mark fish prior to cohabitation for challenge. The results demonstrated significant protection against all challenge isolates, and relative percent survivals ranged from 79% to 100%. SDS-PAGE analysis of whole-cell lysate proteins from the *S. iniae* isolates demonstrated similar protein profiles between 10 and 31 kDa and variation in profiles between 35 and 100 kDa. Western blot analysis using antiserum from vaccinated fish (ARS-98-60) demonstrated shared immunogenic proteins among all isolates in the molecular mass range of 22–35 kDa and high molecular mass material >150 kDa. The results suggest that the developed *S. iniae* vaccine has broad ranging

protection among isolates exhibiting different protein profiles.

**Keywords:** bacterin vaccine, *Oreochromis niloticus*, protein profiles, *Streptococcus iniae*, Western blot.

### Introduction

*Streptococcus iniae* is a significant aquatic animal pathogen in farmed and wild fish (Shoemaker, Klesius & Evans 2001; Klesius, Evans, Shoemaker & Pasnik 2006a; Agnew & Barnes 2007; Klesius, Shoemaker & Evans 2008). The losses in aquaculture production systems have been reported to range from 30% to 75% dependent on culture system (Eldar, Bejerano & Bercovier 1994; Perera, Johnson, Collins & Lewis 1994; Stoffregen, Backman, Perham, Bowser & Babish 1996). Control strategies include management practices (e.g. removal of dead/moribund fish, reduction of stocking rates), chemotherapeutants (e.g. antibacterials) and prevention (e.g. vaccination).

Killed autogenous bacterins were developed and successfully used in Israel (Bercovier, Ghittino & Eldar 1996; Eldar, Horovitz & Bercovier 1997). More recently, it has been reported that these vaccines have failed because of the emergence of a new isolate (serotype) (Zlotkin, Hershko & Eldar 1998; Bachrach, Zlotkin, Hurvitz, Evans & Eldar 2001; Eyngor, Tekoha, Shapira, Hurvitz, Zlotkin, Lublin & Eldar 2008). The autogenous strategy has also been employed in Australia; however, this strategy has yielded mixed results. It has been reported that disease reoccurs in vaccinated fish (Creeper & Buller 2006) and serological diversity among the isolates has been suggested as the reason

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for the lack of vaccine efficacy (Agnew & Barnes 2007). Two other vaccines have recently been marketed for use against *S. iniae*, both offered by Intervet Schering Plough (Agnew & Barnes 2007) but have limited geographical availability. In addition to bacterins, attenuated strains of *S. iniae* have been produced and are now being evaluated as modified live vaccines (Buchanan, Stannard, Lauth, Ostland, Powell, Westerman & Nizet 2005; Locke, Aziz, Vicknair, Nizet & Buchanan 2008). However, there may be difficulty with regulatory approval of these vaccines as *S. iniae* is emerging as a zoonotic pathogen (Weinstein, Litt, Kertesz, Wyper, Rose, Coulter, McGeer, Facklam, Ostach, Willey, Borczyk & Low 1997; Fuller, Darrin, Nizet, Low & DeAzavedo 2001; Lau, Woo, Tse, Leung, Wong & Yuen 2003; Facklam, Elliott, Shewmaker & Reingold 2005; Lau, Woo, Luk, Fung, Hui, Fong, Chow, Wong & Yuen 2006).

Our laboratory developed a modified killed bacterin vaccine to prevent *S. iniae* disease in Nile tilapia, *Oreochromis niloticus* (L.) (Klesius, Shoemaker & Evans 1999, 2000). The vaccine is based on the addition of killed whole cells to concentrated extracellular products (ECP) obtained after 72-h fermentation. Following intraperitoneal (IP) injection, the vaccine stimulates protective antibodies that have been shown to be important in protective immunity (Shelby, Klesius, Shoemaker & Evans 2002a). Further, the ECP have been shown to stimulate movement of macrophages *in vitro*, thus suggesting the initiation of the inflammatory response (Klesius, Evans & Shoemaker 2007). The initial vaccine tested was developed with a *S. iniae* isolate (ARS-98-10) obtained from a diseased tilapia. Interestingly, challenge with the homologous isolate resulted in lower protection than was demonstrated following challenge with a heterologous isolate (ARS-98-60) (Klesius *et al.* 2000). We further demonstrated that immunization with both isolates (ARS-98-10 and ARS-98-60) resulted in improved protection [relative percent survivals (RPS) of 63–87%]; however, no protein or Western blot analysis was completed. Consistent vaccine efficacy is obtained in the laboratory using the vaccine incorporated with the ARS-98-60 isolate, and RPS values of 80–100% are commonly documented following IP administration and homologous isolate (ARS-98-60) challenge (Klesius, Evans, Shoemaker & Pasnik 2006b; Shoemaker, Vandenberg, Desormeaux, Klesius & Evans 2006). The objectives of

this study were to determine whether the developed modified *S. iniae* bacterin vaccine is efficacious against challenge with heterologous isolates and to evaluate protein and antigenic variability among the isolates tested.

## Materials and methods

### Bacteria

Five *S. iniae* isolates obtained from different fish species exhibiting clinical streptococcal disease and from different geographical regions were used in the present study (Table 1). The isolates were identified by standard methods (Shoemaker & Klesius 1997) and by API 20 Strep test (BioMerieux). The archived isolates were recovered from frozen stocks (2 mL aliquots stored at  $-80^{\circ}\text{C}$ ) and grown in tryptic soy broth (TSB; Difco Laboratories) for 24 h at  $28^{\circ}\text{C}$ . All isolates were passed through Nile tilapia once and reisolated in pure culture on sheep blood agar (SBA; Remel) prior to inoculation into TSB for use in bacterial challenges and extraction of whole-cell lysate proteins. Isolates were grown for 24 h at  $28^{\circ}\text{C}$  and adjusted to an optical density (OD) of 1.0 at 540 nm. Samples of the inocula were plated onto SBA plates to confirm purity, and the number of colony-forming units (CFU)  $\text{mL}^{-1}$  of each isolate were determined by plating tenfold serial dilutions on SBA plates using standard procedures.

### Nile tilapia

Three hundred and twenty Nile tilapia obtained from stocks maintained at USDA-ARS, Aquatic Animal Health Research Laboratory (Auburn, AL, USA) were used. Tilapia (mean weight  $5.45 \pm 0.3$  g) were acclimated in flow-through 57-L aquaria supplied with  $0.5 \text{ L h}^{-1}$  dechlorinated water for 10 days prior to experiments. A light:dark

**Table 1** *Streptococcus iniae* isolates used in this study

Isolate designation (year isolated)	Location	Species of fish
ARS-98-60 (1998)	California, USA	<i>Morone saxatilis</i> × <i>M. chrysops</i>
08-9698 (2008)	Hawaii, USA	<i>Seriola rivoliana</i>
MN-15B (2007)	Minnesota, USA	<i>Oreochromis niloticus</i> × <i>O. aureus</i>
BZ-1 (2005)	Brazil	<i>Oreochromis niloticus</i>
ID-35-B (2007)	Idaho, USA	<i>Oreochromis</i> spp.

period of 12:12 h was maintained, and supplemental aeration was supplied by an air stone. The fish were fed daily (approximately 3% body weight) with Aquamax Grower 400 (Purina). To verify the *S. iniae*-free status of the fish, samples were obtained for bacterial culture by passing an inoculation loop into the brain and kidney of 20 fish sampled from the population of origin. None of the fish sampled were found to be culture positive for *S. iniae* following incubation of the samples on SBA plates for 24 h. The dissolved oxygen, temperature, pH, salinity, hardness, ammonia and nitrites were measured using a CEL/890 Advanced Portable Laboratory (Hach). During the experiment, the mean dissolved oxygen was 5.6 mg L<sup>-1</sup>, temperature was 26 °C, pH was 7.1 and hardness was 100 mg L<sup>-1</sup>. Ammonia and nitrite concentrations (mg L<sup>-1</sup>) were below the detection limits.

#### Vaccine preparation and administration

Vaccine was prepared as previously described by Klesius *et al.* (1999, 2000). Briefly, *S. iniae* (ARS-98-60) was static cultured in TSB for 72 h at 28 °C. The culture was then treated for 24 h with 10% neutral buffered formalin to give a final concentration of 3%. The formalin-treated culture was centrifuged at 7000 g for 30 min, and the cell pellet and supernatant were separated. The cell-free supernatant was concentrated 20-fold using a 2 kDa spiral concentrator (Amicon, Inc.), filtered sterilized (0.2 µm), and used to resuspend the cell pellet at a ratio of 10:1 (v/v). The final cell concentration of the vaccine was approximately 4 × 10<sup>9</sup> CFU mL<sup>-1</sup> (based on previous plate counts). The vaccine was considered killed if there was no growth on SBA following 72-h culture at 28 °C. The vaccine was administered by IP injection in a volume of 100 µL, and sham-vaccinated (control) fish were IP injected with the same volume of sterile TSB. Vaccinated fish (160) were housed in four 57-L aquaria, and 160 sham-vaccinated fish were held in four other aquaria prior to calcein marking (see below).

#### Calcein marking and *Streptococcus iniae* challenge

Calcein (Sigma Chemical) was dissolved in water at a concentration of 500 mg L<sup>-1</sup> (Klesius *et al.* 2006b). A total of 160 sham-vaccinated control fish were calcein marked (CM) for use in the

challenge. Eighty sham-vaccinated tilapia were immersed in each of two tanks containing 10 L calcein solution for 4 h with aeration and returned to aquaria with flow-through water for 24 h to remove excess calcein. Eighty vaccinated tilapia were immersed in each of two tanks containing 10 L water without addition of calcein (non-marked, NM) to mimic handling stress. Seventy-two hours following marking and 28 days post-vaccination, vaccinated and sham-vaccinated tilapia were challenged with each of the four heterologous *S. iniae* isolates by IP injection with 100 µL volumes containing 2–3 × 10<sup>7</sup> CFU per fish. For each isolate, four replicate aquaria were used; each containing ten sham-vaccinated (CM) and ten vaccinated (NM) tilapia (20 fish total per replicate). Dead and moribund fish were collected daily for 14 days and examined for calcein marks using a plug-in mineral lamp Model UVGL-58 (Ultra-Violet Products) to distinguish between vaccinated or sham-vaccinated fish as described by Klesius *et al.* (2006b). Dead and moribund fish were also cultured and the bacteria isolated were identified using standard microbiology to confirm *S. iniae*. The mean cumulative percent mortality for sham-vaccinated and vaccinated tilapia for each replicate and challenge isolate was calculated, and RPS was determined (Amend 1981).

Prior to bacterial challenge (at time of calcein marking), sham-vaccinated and vaccinated tilapia (*n* = 18 per group) were non-lethally bled by caudal puncture using a 26-gauge needle. Post-challenge, 2–4 fish were bled from each replicate per isolate group using the same procedure. Blood was transferred to microcentrifuge tubes, allowed to clot for 2 h at room temperature and centrifuged at 8000 g for 10 min. Serum was collected and stored at –20 °C.

#### Indirect enzyme-linked immunosorbent assay (ELISA)

Antibody titre against *S. iniae* was determined (prior to and following challenge) using the method described by Shelby, Shoemaker & Klesius (2002b). Briefly, 96-well ELISA plates were coated for 1 h at 25 °C with 100 µL *S. iniae* (ARS-98-60) antigen in carbonate buffer (CB), which was obtained following sonication and size-exclusion chromatography of *S. iniae* (a 1:10 dilution in CB of the initial fraction, which represented the highest molecular weight fraction, was used). Plates were

washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and then blocked with 3% bovine serum albumin in CB for 1 h. Following blocking, plates were washed three times with PBS-T. Tilapia serum was then added at a 1:20 dilution in PBS-T and serially diluted down each column of the plate. Serum was incubated for 30 min at 25 °C and then plates were washed three times with PBS-T. Monoclonal anti-tilapia immunoglobulin (1H1; Shelby *et al.* 2002b) was diluted 1:1000 in PBS-T and then added to all wells (100 µL per well) for 30 min. Following washing three times with PBS-T, 100 µL of sheep anti-mouse IgG peroxidase conjugate (1:5000 in PBS-T) was added and incubated for 15 min. Plates were washed as described earlier and 100 µL substrate was added (tetramethylbenzidine; Pierce). The reaction was stopped after 15 min by adding 50 µL of 3 M H<sub>2</sub>SO<sub>4</sub> to each well and the OD was read at 450 nm using a spectrophotometer. Serum antibody endpoint titres were reported as the reciprocal of the last dilution yielding an OD more than twice that of the control values (i.e. OD of negative serum). *S. iniae*-positive and *S. iniae*-negative sera were included on each plate as assay controls.

#### Preparation of whole-cell lysates and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Whole-cell lysates of each *S. iniae* isolate were prepared using a method modified from Encheva, Gharbia, Wait, Begum & Shah (2006). Approximately 200 mg (wet weight) of cells were resuspended into 1 mL of lysis buffer (0.2% SDS, 133 mM DTT, 17 mM MgCl<sub>2</sub>, 50 mM Tris) containing 375 U of benzonase (Sigma) and 350 U of mutanolysin (Sigma). The cells were incubated overnight at 37 °C then transferred to Lysing Matrix B tubes (MP Biomedicals) and homogenized for 30 min in a FastPrep<sup>®</sup>-24 (MP Biomedicals) instrument with cooling on ice every 2 min. Tubes were centrifuged at 16 000 g for 30 min at 4 °C, and the supernatant was collected and stored at –80 °C. For each isolate, the proteins contained in a 100 µL aliquot were precipitated using trichloroacetic acid/acetone and resuspended into an equal volume of PBS to remove contaminants that interfere with standard protein assays. The protein concentration was determined using a Micro BCA<sup>™</sup> Protein Assay (Pierce).

Whole-cell lysates from each isolate were analysed by SDS–PAGE as described by LaFrentz, LaPatra, Jones & Cain (2004). Proteins (25 µg) were separated in precast 12% polyacrylamide gels using a Mini-Protean Tetra Cell (Bio-Rad) and then stained with Bio-Safe Coomassie (Bio-Rad). Precision Plus protein standards (Bio-Rad) were used to estimate the molecular mass of protein bands. Gels were digitally imaged using a GS-710 Calibrated Imaging Densitometer (Bio-Rad).

#### Western blot analysis

Following SDS–PAGE, proteins from each isolate were transferred to nitrocellulose membranes by electrophoresis at 100 V for 1 h using a Mini Trans-Blot<sup>®</sup> Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's directions. The membranes were blocked for 1 h with tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.4) containing 0.05% Tween-20 and 10% non-fat dry milk (TBST-NFDM) at room temperature. Sera from vaccinated and sham-vaccinated tilapia (pools of equal volumes from 13 fish prior to challenge) were diluted 1:50 into TBST-NFDM, applied to the membranes and incubated at 4 °C overnight on an orbital shaker. The tilapia antibody was detected by incubation of the membranes for 1 h at room temperature with a monoclonal antibody specific for tilapia antibody (MAb 1H1, Shelby *et al.* 2002b) diluted 1:500 in TBS containing 0.05% Tween-20 (TBST). Membranes were then incubated for 1 h with an alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) antibody (Sigma) diluted 1:5000 in TBST. Between each incubation, membranes were washed three times for 5 min in TBST. The immunoreactive bands were visualized using an AP conjugate substrate kit (Bio-Rad) and digitally imaged using a GS-710 Calibrated Imaging Densitometer (Bio-Rad).

#### Statistical analysis

Mean cumulative mortality data between sham-vaccinated and vaccinated tilapia were compared using one-way analysis of variance and the TTEST procedure (SAS) to determine significant ( $P < 0.05$ ) differences. Mean ELISA titres were compared by GLM procedure, and Duncan's multiple range test was used to determine significant differences ( $P < 0.05$ ) between means (SAS).

## Results

### API 20 results

API 20 strep strips resulted in similar codes at 24 h for ARS-98-60, MN-15B, BZ-1 (API 24 h – 4561117). The isolates were positive for esculin, pyrrolidonyl arylamidase,  $\beta$ -glucuronidase, alkaline phosphatase, leucine aminopeptidase, arginine dihydrolase (ADH), mannitol, trehalose, starch and glycogen. Isolate ID-35-B was ADH negative and glycogen negative (API 24 h – 4560115). Isolate 08-9698 was positive in all reactions except mannitol at 24 h (API 24 h – 4561017).

### Vaccine trial

Mean cumulative percent mortality of ARS-98-60 vaccinated tilapia challenged with each of the respective isolates was significantly less ( $P < 0.05$ ) than mean cumulative percent mortality determined for the equivalent cohoused sham-vaccinated tilapia (Table 2). RPS ranged from 79% to 100% at 14 days post-challenge. All dead tilapia were cultured and confirmed *S. iniae* positive by standard microbiological methods (data not shown).

### Indirect ELISA

At 25 days post-vaccination (prechallenge), vaccinated tilapia had significantly higher ( $P < 0.05$ ) mean antibody titres ( $703 \pm 244$ ) than sham-vaccinated tilapia ( $36 \pm 5$ ) (Table 3). Post challenge, the antibody titres in all treatment groups regardless of challenge isolate were similar (Table 3). These antibody titres were similar to

**Table 2** Mean cumulative percent mortality (CPM) and relative percent survival (RPS) of ARS-98-60 vaccinated tilapia challenged with different isolates using the calcein cohabitation model (Klesius *et al.* 2006b)

Challenge isolate	Mean CPM <sup>1</sup> of sham-vaccinated tilapia $\pm$ SE	Mean CPM of vaccinated tilapia $\pm$ SE	RPS
08-9698	42.5 $\pm$ 4.8 <sup>a</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	100
MN-15B	35.0 $\pm$ 8.6 <sup>a</sup>	7.5 $\pm$ 4.8 <sup>b</sup>	79
BZ-1	32.5 $\pm$ 2.5 <sup>a</sup>	2.5 $\pm$ 2.5 <sup>b</sup>	92
ID-35-B	17.5 $\pm$ 2.5 <sup>a</sup>	2.5 $\pm$ 2.5 <sup>b</sup>	86

Different letters within a row indicate significant differences ( $P < 0.05$ ) in cumulative percent mortality by paired *t*-tests between sham-vaccinated and vaccinated tilapia.

<sup>1</sup>Each mean is based on four replicated aquaria of sham-vaccinated (ten calcein marked) and vaccinated (ten non-marked) tilapia.

**Table 3** Serum antibody titres of tilapia post-vaccination and post-heterologous *Streptococcus iniae* isolate challenge

Treatment	N	Range of titres	Mean serum antibody titre $\pm$ SE <sup>1</sup>
Post-vaccination			
Vaccinated	18	40–2560	703 $\pm$ 244 <sup>a</sup>
Sham-vaccinated	18	20–80	36 $\pm$ 5 <sup>b</sup>
Post-challenge <sup>2</sup>			
08-9698	8	80–1280	650 $\pm$ 186
MN-15B	16	40–2560	1080 $\pm$ 177
BZ-1	16	320–2560	1170 $\pm$ 274
ID-35-B	16	40–2560	952 $\pm$ 250

Means with different superscripts indicate significant difference ( $P < 0.05$ ) in mean antibody titre between vaccinated and sham-vaccinated fish prior to challenge (25 days post-vaccination).

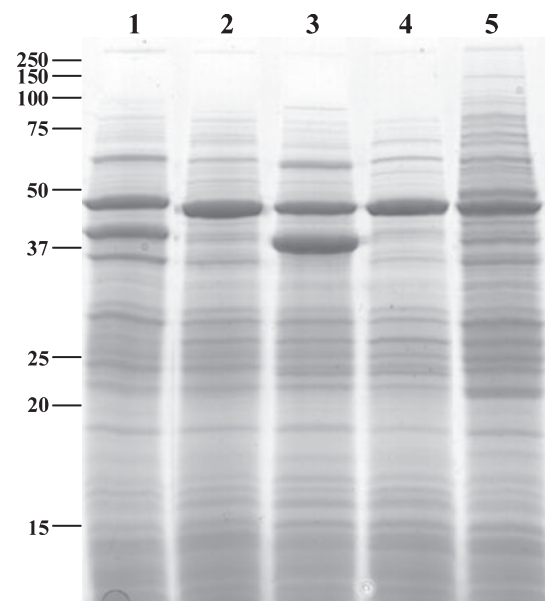
<sup>1</sup>SE = standard error.

<sup>2</sup>Mean antibody titres were not significantly ( $P > 0.05$ ) different post-challenge.

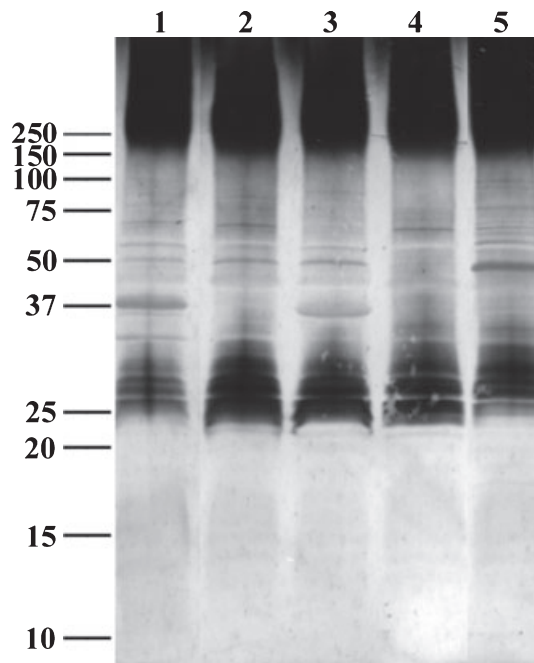
those of the vaccinated tilapia measured at day 25 (i.e. prechallenge).

### Protein profiles and Western blot

SDS–PAGE analysis of whole-cell lysate proteins from the *S. iniae* isolates demonstrated similar protein profiles between 10 and 31 kDa with some variability in the staining intensity of bands (Fig. 1). A larger degree of protein variation was observed in the molecular mass range of



**Figure 1** Protein profiles of *Streptococcus iniae* isolates. Lane (1) BZ-1; Lane (2) MN-15B; Lane (3) 08-9698; Lane (4) ID-35-B; Lane (5) ARS-98-60. Molecular mass markers (kDa) are indicated on the left of the gel.



**Figure 2** Western blot analysis of *Streptococcus iniae* isolates. Antiserum used for blotting was from ARS-98-60 vaccinated fish. Lane (1) BZ-1; Lane (2) MN-15B; Lane (3) 08-9698; Lane (4) ID-35-B; Lane (5) ARS-98-60. Molecular mass markers (kDa) are indicated on the left of the blot.

35–100 kDa. Western blot analysis using antiserum from vaccinated fish (ARS-98-60) demonstrated shared immunogenic proteins among all isolates in the molecular mass range of 22–35 kDa and high molecular mass material > 150 kDa (Fig. 2). Considerable variability was observed in immunoreactive proteins between 37 and 100 kDa depending on the isolate. Sera from sham-vaccinated tilapia exhibited slight immunoreactivity to a few *S. iniae* proteins (data not shown).

## Discussion

Efficacy against four heterologous farm isolates of *S. iniae* obtained from diverse geographical locations was demonstrated following immunization with the *S. iniae* (ARS-98-60) modified bacterin vaccine. The vaccine isolate was obtained from a diseased hybrid striped bass, *Morone saxatilis* × *M. chrysops*. Three of the four heterologous isolates were obtained from diseased tilapia, *Oreochromis* sp., grown in intensive culture systems. The fourth isolate was from a diseased Almaco jack, *Seriola rivoliana* Valenciennes, cultured in open

ocean salt water cages. Biochemically, using API 20 strep strips, the isolates were similar at 24 h with the exception of 08-9698 and ID-35-B. The 08-9698 isolate was negative for mannitol acidification. ID-35-B was negative for ADH and glycogen acidification. ADH-negative isolates (using API 20 strep) are considered serotype II *S. iniae* (Bachrach *et al.* 2001; Barnes, Young, Horne & Ellis 2003; Shin, Palaksha, Kim, Nho, Kim, Heo, Park & Jung 2007) and ADH-positive isolates are serotype I (Bachrach *et al.* 2001; Barnes *et al.* 2003) using antiserum generated in trout. Barnes & Ellis (2003) have shown in standard tube tests that API 20 strep ADH-positive and ADH-negative strains both have the ability to hydrolyse arginine. All isolates used in this study were positive by tube test (data not shown).

Limited work has been conducted on the protein and antigenic diversity of *S. iniae*. Barnes *et al.* (2003) suggested that no difference was observed by SDS-PAGE analysis of outer membrane or whole-cell protein profiles of different *S. iniae* isolates. These authors suggested that serologic differences were observed in rainbow trout, *Oncorhynchus mykiss* (Walbaum), using agglutination analysis with serum obtained following immunization with formalin-fixed bacterins in adjuvant. The serologic types were similar to those suggested previously by Zlotkin *et al.* (1998). Our results suggest that while protein profiles were somewhat similar, each isolate presented a unique pattern, especially in the 37–50 kDa range. This pattern (i.e. differences between 20 and 62 kDa) was similar to that reported by Shin, Palaksha, Yang, Shin, Kim, Lee, Kim, Kim, Oh, Yoshida & Jung (2006) studying isolates obtained from olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). Shin *et al.* (2006) is also one of the few articles to present Western blot results; however, they used antisera raised against *Lactococcus garvieae* in chickens. Regardless of the antiserum, the results of Shin *et al.* (2006) suggested minor banding differences between the *S. iniae* isolates used. In our study, we performed Western blot analysis using serum obtained from vaccinated tilapia, and the results demonstrated that each isolate presented blotting patterns different from the vaccine isolate. However, the isolates did share immunogenic components consisting of high molecular mass (> 150 kDa) material as well as low molecular mass bands (< 35 kDa).

Shin *et al.* (2007) used ECP vaccine formulated using the cellophane overlay technique in olive flounder. These authors demonstrated protection against a high and moderately virulent isolate if the ECP was mixed with the formalin-fixed cells. They suggested that the difference in virulence was attributed to the amount of ECP secreted. Using 2D-electrophoresis, these authors demonstrated similarities in proteins of the ECP between the two isolates tested if grown for longer than 48 h. They also showed protection against both isolates if ECP was delivered in conjunction with the cells from the high virulent isolate (Shin *et al.* 2007). Although the protective components of the modified bacterin vaccine developed in our laboratory have not been described, the ability of the vaccine to protect against the four heterologous isolates may be attributed to the use of long-term fermentation (72 h), thus allowing sufficient secretion of protective components, such as the capsular polysaccharide associated with the ECP (Kanai, Notohara, Kato, Shutou & Yoshikoshi 2006; Eyngor *et al.* 2008). Additionally, the antibodies induced following vaccination against the shared immunogenic components among the isolates may be important for the cross protective efficacy demonstrated. Previous work has demonstrated that anti-*S. iniae* antibodies are important for protection, either directly via passive immunization (Shelby *et al.* 2002a) and/or indirectly via elevated antibody levels prior to challenge (Eldar *et al.* 1997; Klesius *et al.* 2000). In the present study, vaccinated fish had elevated antibody titres prior to challenge and the anti-*S. iniae* ARS-98-60 sera from the vaccinated fish recognized antigens from the different isolates by Western blot, further supporting the role of specific antibody in protection.

### Acknowledgements

The authors thank Paige Mumma and Alberta Pink and the animal care staff for their technical contributions. We also thank Dr David Pasnik and Dr Julia Pridgeon for critically reading the manuscript. This work was supported by USDA-ARS CRIS No. 6420-32000-019-00D. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply endorsement by the United States Department of Agriculture.

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Received: 24 August 2009

Revision received: 16 December 2009

Accepted: 7 January 2010